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Exposure to microplastics reduces attachment strength and alters the haemolymph proteome of blue mussels (*Mytilus edulis*)

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Abstract

The contamination of marine ecosystems with microplastics, such as the polymer polyethylene, a commonly used component of single-use packaging, is of global concern. Although it has been suggested that biodegradable polymers, such as polylactic acid, may be used to replace some polyethylene packaging, little is known about their effects on marine organisms. Blue mussels*, Mytilus edulis*, have become a "model organism" for investigating the effects of microplastics in marine ecosystems. We show here that repeated exposure, over a period of 52 days in an outdoor mesocosm setting, of *M. edulis* to polyethylene microplastics reduced the number of byssal threads produced and the attachment strength (tenacity) by ~50%. Exposure to either type of microplastic altered the haemolymph proteome and, although a conserved response to microplastic exposure was observed, overall polyethylene resulted in more changes to protein abundances than polylactic acid. Many of the proteins affected are involved in vital biological processes, such as immune regulation, detoxification, metabolism and structural development. Our study highlights the utility of mass spectrometry-based proteomics to assess the health of key marine organisms and identifies the potential mechanisms by which microplastics, both conventional and biodegradable, could affect their ability to form and maintain reefs.

Capsule

Conventional microplastics alone reduced the attachment strength of blue mussels but both conventional and biodegradable micoplastics altered the haemolymph proteome.

Keywords

Biodegradable microplastics, tenacity, proteins, polylactic acid, polyethylene, immunity.

1. Introduction

Microplastic particles (0.1 µm – 5 mm) are the most numerically abundant form of solid waste on Earth (Eriksen et al. 2014) and are a potential threat to marine ecosystems globally (Galloway et al. 2017). Global plastic production, which has risen from ~1.5 million metric tonnes (MTT) in 1950 to ~335 MMT in 2016, is predicted to continue to rise (Plastics Europe, 2017). The majority (39.9%) of this production is designed for use in single-use packaging, most of which is composed of polyethylene (PE) (Plastics Europe, 2017). Plastics that are able to biodegrade in composting facilities ("biodegradable"), however, have been proposed as suitable alternatives to conventional packagings, such as PE, and global production for these polymers is also growing (Bioplastics Europe, 2016). Polylactic acid (PLA), derived from e.g. starch, accounts for ~45% of the global market of biodegradable plastics and ~60% of its production is used to make packaging (Markets and Markets, 2015). Most microplastics arise from the fragmentation of larger plastic litter items rather than being directly littered as micro-sized particles (for example, microbeads). Packaging items are also the most abundant form of coastal litter (Galgani et al. 2015) and are, therefore, likely the greatest source of microplastic litter.

Current estimates of environmental concentrations of microplastics in coastal waters are biased towards larger particles because the mesh sizes used to sample are generally > 330 µm. When a smaller mesh is used, however, estimates of concentrations in seawater have been found to be three orders of magnitude greater (e.g. from ~0.005 L^{-1} with a 335 µm net to up to ~6 L^{-1} with grab samples filtered over a 0.45 µm filter; Barrows et al. 2017). Given that global production of waste is not expected to peak before 2100 and that the amount of plastic in the marine environment is expected to increase by an order of magnitude by 2025

75 (Jambeck et al. 2015), abundances of microplastics, and their potential impacts on marine
76 organisms, are likely to be even greater in the coming decades.
77
78 Understanding the impacts of microplastics is ongoing and has been the subject of several
79 recent reviews (such as Galloway et al. 2017, de Sá et al. 2018 and Anbumani and Kakkar
80 2018). Microplastics have been shown to negatively affect neural (neurotoxicity in
81 nematodes; Lei et al 2018), cellular (oxidative stress in leucocytes of gilthead seabream;
82 Espinosa et al. 2018) to population level (reduced reproductive output of oysters; Sussarellu
83 et al. 2016) biological processes and functions. One of the most well studied marine
84 organisms with respect to microplastics, is the blue mussel, *Mytilus edulis*. *M. edulis* is an
85 important ecosystem engineer, clinging together using byssal threads (extensible
86 proteinaceous fibres; Waite, 1991) to form reefs, which are important biogenic habitats (Seed,
87 1996). They are filter feeders and have been found to ingest microplastics in natural (Van
88 Cauwenberghe et al. 2015; De Witte et al. 2014) and experimental settings (Browne et al.
89 2008; von Moos et al. 2012). Despite this growing body of work, our understanding of how
90 microplastics affect the general health of *M. edulis*, ranging from its biological activity to its
91 underlying molecular phenotype, is currently lacking.
92
93 Haemolymph is the blood-plasma equivalent of the invertebrate circulatory system, involved
94 in the transportation of oxygen, nutrients, as well as proteins involved in important biological
95 processes, including the nervous system, reproduction and the immune system. Within
96 molluscs, the haemolymph has been a well-studied medium for understanding the immune
97 complement, as well as functionality, including aspects of both the cellular (Carballal et al.
98 1997) and humoral immune response (Campos et al. 2015). Directly assessing the cellular
99 and molecular composition of haemolymph, particularly proteins, provides an insight into the

general health of an invertebrate organism and can reveal the signatures of environmental stress (Brown et al. 2006; Hannam et al. 2010). Invertebrate haemolymph protein abundances may be assessed using enzyme assays, gel electrophoresis or directly using mass spectrometry-based proteomics, which results in the unbiased identification and quantification of multiple proteins in a given sample (e.g. Campos et al. 2015; McNamara et al. 2017). The increasing incorporation of -omics techniques within the study of key ecological species have improved our understanding of the immune complement (Campos et al. 2015; Wu et al. 2016), structural composition (Mann et al. 2012; Mann and Edsinger, 2014), biomarkers of disease and pollution (Wu et al. 2016; Campos et al. 2016) as well as responses of organisms to environmental change (Tomanek 2012; Tomanek 2014).

To date, the vast majority of experiments investigating detrimental effects of microplastics exposure have used highly controlled laboratory conditions, with very high concentrations of microplastics, for example 110 (Van Cauwenberghe et al. 2015) to >1 billion (Browne et al. 2008) particles ml^{-1}, which do not simulate natural conditions of the habitats in which the organisms reside. Compared with indoor laboratories, outdoor mesocosm systems can provide a better understanding of environmentally relevant effects of contaminants on individuals, populations and communities (Miko et al. 2015). In order to estimate future risks, an outdoor mesocosm experiment was used to expose *M. edulis* to a relatively low concentration (~1 particle mL^{-1} or 25 µg L^{-1}) of a range of particle sizes (~0.5 to ~330 µm) of conventional (high-density polyethylene - HDPE) or biodegradable (polylactic acid - PLA) microplastics in a natural outdoor setting for 52 days to assess impacts on (i) important biological traits as ecosystem engineers including byssal thread production and attachment strength and (ii) underlying molecular health through proteomic analysis of their haemolymph.

2. Methods

2.1. Experimental design and set-up

Mesocosm experiments were set up at the outdoor flow-through mesocosm facility at Queen's University Marine Laboratory, Portaferry, Northern Ireland. The experimental design consisted of one fixed factor "Plastic", with three levels: no microplastics added (control), polylactic acid (PLA) and high density polyethylene (HDPE).

Full details of the experimental set-up can be found in Green et al. (2017), but are described here in brief. Mesocosms were 10 litre polypropylene buckets (height x diameter = 25 x 25 cm). The experiment had 5 replicates (n = 5, N = 15) and to simulate the habitat where the mussels were collected from, each mesocosm contained a 5 cm layer of muddy sediment. On the 26th of August 2014, after allowing 48 hours for the sediment to settle, each mesocosm received 7 individuals of *M. edulis*. Mussels were measured, weighed and randomly allocated to treatments in order to ensure that no biases due to size were introduced into the experiment. The average (±S.E.M.) whole animal wet weight was 19.84 ± 0.51 g and there were no differences among treatments (ANOVA: $F_{2,12}$ = 0.18, P = 0.831). The average (±S.E.M) length of the mussels was 48.17 ± 0.83 mm and there were also no differences in length among treatments (ANOVA: $F_{2,12}$ = 0.87, P = 0.469). The experiment ran for 52 consecutive days (until 14th October 2014). Sand-filtered seawater, sourced from Strangford Lough (54°22'51.1"N; 5°33'04.0"W) was continuously provided to each mesocosm at constant flow rates (~500 mL minute^{-1}) via individual hoses resulting in an overlying water column of ~8 L.

2.2. Microalgal culture and microplastic dosing

Cultures of the microalgae *Isochrysis galbana* were prepared using seawater (35 psu), which was filtered with 0.45 μm aperture membranes and sterilised with UV light. PLA or HDPE microplastics were then added to 5 litre bottles with *I. galbana* and were continuously mixed with air bubblers. Fresh batches of control and microplastic-dosed *I. galbana* cultures were made weekly. Virgin (pre-production) white PLA and HDPE microplastic particles (fragments not spheres) used in the experiments had a volume-weighted mean diameter of 65.6 μm (range = 0.6–363 μm) for PLA and 102.6 μm (range = 0.48–316 μm) for HDPE. Every day, each mesocosm received 250 mL of ~2×10^6 cells mL^{-1} of microalgae containing either 0 (control) or 800 μg L^{-1} of PLA or HDPE microplastics, equating to a concentration in the mesocosms of 25 μg L^{-1}. This corresponds to approximately 1296.3 (± 182.9) and 844.9 (± 138.7) particles L^{-1} respectively (Green et al. 2017). Although this is high relative to current environmental concentrations, due to the range of particle sizes included, these concentrations are among the lowest used experimentally to date and, therefore, among the most environmentally realistic (Lenz et al. 2016). In order to simulate a pulse of microplastics in the environment (such as from wastewater effluent), mussels were exposed for two hours per day. During dosing, the flow of water was paused and air bubblers were used to keep the water moving and aerate the mesocosms. The water flow in the mesocosms was resumed after 2 hours and fresh seawater replaced the microplastic- contaminated water which was captured for safe disposal.

2.3. Tenacity of mussels

The remaining individuals from each of the mesocosms (excluding those removed for filtration and proteomics measurements) were placed onto glass petri dishes in clean buckets with fresh flowing seawater. Feeding (without microplastics) was resumed for a further 3 days (in order to allow the mussels to attach their byssal threads) before tenacity was

measured for one randomly selected mussel from each replicate mesocosm. Tenacity was measured following the method of Denny (1987). Briefly, a portable dynometer (Pesola, Sweden), scaled 0 to 10 N, was used to measure the maximal vertical force required for the individual to become dislodged (attachment strength, N). The dynometer had a small clamp that gripped individual mussels. The maximum dislodgement force was marked by a ring on the dynamometer, which remained in place after detachment indicating the maximum extension of the spring (to the nearest 0.1 N). Shell planform area (A_{pl}) was approximated as an ellipse with shell height and width as major and minor axes (measured with vernier callipers to 1 mm), respectively (Bell and Gosline 1997). Tenacity was calculated as dislodgement force per unit mussel planform area, in N m^{-2}. The number of byssal threads deployed by each of the mussels used in tenacity measurements was also counted.

2.4. Proteomic analysis of mussels

After 50 days, haemolymph was extracted from one individual from each mesocosm. In order to minimise disturbance, exposure to the air was not longer than 3 minutes before haemolymph extraction. Prior to haemolymph extraction, the shells were temporarily opened 2-3 mm to allow for the removal of excess water from the interior. The shells were then allowed to reclose and form a tight seal. An Omnican F syringe with an integrated 30 gauge needle was inserted between the shells of the mussel into the posterior adductor muscle and haemolymph slowly collected. The syringe was then removed from the needle and the haemolymph was dispensed into a nuclease-free 1.5 ml microcentrifuge tube. The haemolymph was stored temporarily at -20°C overnight before transfer to -80°C for long-term storage. As haemocytes can be reactive to oxygen causing the production of melanin, we took additional care to remove haemocytes prior to sample preparation for mass-spectrometry.

200 200 µl crude haemolymph was removed and centrifuged at 8,000 × g for 5 min to pellet
201 cellular debris. The supernatant was then removed, quantified and four independent biological
202 replicates were analysed from each treatment: control (n=4), PLA- (n=4) and HDPE-exposed
203 (n=4) mussels. 50 µg of each sample was precipitated using the 2D Clean-Up Kit (GE
204 HealthCare), following the manufacturer's instructions and the resulting protein pellet was
205 resuspended in 50 µl 6M urea, 2M thiourea, 0.1 M Tris-HCl, pH 8.0. 50mM ammonium
206 bicarbonate was added to each sample and proteins were reduced with 0.5M dithiothreitol
207 (DTT) at 56°C for 20 min and alkylated with 0.55M iodoacetamide (IAA) at room
208 temperature for 15 min, in the dark. 1 µl of a 1% w/v solution of Protease Max Surfactant
209 Trypsin Enhancer (Promega) and 0.5 µg of Sequence Grade Trypsin (Promega) was added to
210 give a protein:trypsin ratio of 100:1. The protein/trypsin mixture was incubated at 37°C for
211 18 h. Digestion was terminated by adding 1 µl of 100% trifluoroacetic acid (Sigma Aldrich)
212 and incubation at room temperature for 5 min. Samples were centrifuged for 10 min at 13,000
213 × g and a volume equivalent to 40 µg of pre-digested protein was removed and purified for
214 mass spectrometry using C18 Spin Columns (Pierce), following the manufacturer's
215 instructions. The eluted peptides were dried using a SpeedyVac concentrator (Thermo
216 Scientific Savant DNA120) and resuspended in 2% v/v acetonitrile and 0.05% v/v
217 trifluoroacetic acid (TFA). Samples were sonicated for 5 min to aid peptide resuspension
218 followed by centrifugation for 5 min at 13,000 × g. The supernatant was removed and used
219 for mass spectrometry.
220
221 *2.5. Mass spectrometry*
222 1 µg of each digested sample was loaded onto a QExactive (ThermoFisher Scientific) high-
223 resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano)
224 chromatography system. The peptides were separated by a 4% to 35% gradient of acetonitrile

225 on a Biobasic C18 PicofritTM column (100 mm length, 75 mm ID), using a 55 min reverse-
226 phase gradient at a flow rate of 250 nL min^{-1}. All data were acquired with the mass
227 spectrometer (MS) operating in automatic data dependent switching mode. A full MS scan at
228 140,000 resolution and a scan range of 400-2000 m/z were followed by an MS/MS scan,
229 resolution 17,500 and a range of 200-2000 m/z, selecting the 10 most intense ions prior to
230 MS/MS.
231
232 Protein identification and Label Free Quantification (LFQ) normalisation of MS/MS data
233 were performed using MaxQuant v1.5.6.5 (http://www.maxquant.org) following the general
234 procedures and settings outlined in Hubner et al. (2010). The Andromeda search algorithm
235 (Cox et al. 2011) incorporated in the MaxQuant software was used to correlate MS/MS data
236 against a predicted protein set generated from available RNA sequences for *Mytilus edulis*
237 (Bioproject: PRJEA75259), *Mytilus gallaprovinciallis* (Bioproject: PRJNA167773) and
238 nucleotide/protein sequences available for *M. edulis* and *M. gallaprovinciallis* from the
239 National Centre for Biotechnology and Information (NCBI) repository (8,305 entries,
240 downloaded September 2017) including the predicted protein set derived from the *M.*
241 *gallaprovinciallis* genome (Murgarella) et al. 2016) (Bioproject: PRJNA262617). A 6-frame
242 translation was conducted on the RNA sequences for the transcriptome resources of *Mytilus*
243 species and an open reading frame extraction was performed. CD-Hit (Huang et al. 2010) was
244 utilized to remove redundant sequences that were similar by 98% and above. The final
245 predicted proteome comprised 12,453 sequences and was used in mass spectrometry searches
246 in addition to a contaminant sequence set provided by MaxQuant.
247 The following search parameters were used: first search peptide tolerance of 20 ppm, second
248 search peptide tolerance 4.5 ppm with cysteine carbamidomethylation as a fixed modification
249 and N-acetylation of protein and oxidation of methionine as variable modifications and a

maximum of two missed cleavage sites allowed. False Discovery Rates (FDR) were set to 1% for both peptides and proteins and the FDR was estimated following searches against a target-decoy database. LFQ intensities were calculated using the MaxLFQ algorithm (Cox et al. 2014) from razor and unique peptides with a minimum ratio count of two peptides across samples.

2.6. Processing of proteomic data

Perseus v.1.5.2.6 (www.maxquant.org/) was used for data processing and visualization. The data matrix was first filtered for the removal of contaminants, decoy peptide matches and peptides identified by site. To obtain annotations for all identified proteins a fasta file was generated for the post-filtered proteins using BioEdit (Hall 1999) and BlastP searched using Blast2Go (version: 4.1.9; Conesa et al. 2005) against the NCBI non redundant database with the following search settings: number of blast hits: 20, high-scoring segment pair length cutoff: 33, Blast expect value: $1.0e^{-5}$. These annotations were subsequently uploaded to Perseus. Normalised LFQ intensity values (quantitative measurement of protein abundance) were $\log_2$ transformed and each replicate was assigned to its corresponding sample (n=4). Proteins not found in all 4 replicates of at least one treatment were removed from the analysis. A data-imputation step was conducted to replace non-detected values with values that simulate signals of lowest abundant proteins chosen randomly from a distribution specified by a downshift of 1.8 times the mean standard deviation (SD) of all measured values and a width of 0.3 times this SD. Volcano plots were generated in Perseus by plotting negative log p-values against $\log_2$ fold-change values for each protein to visualize two-fold changes in protein expression of the microplastic exposed mussels relative to the control mussels and microplastic exposed mussels to each other. LFQ intensity values for proteins deemed statistically significant (by ANOVA, see *Statistical data analyses*) were Z-score normalized and hierarchical clustering was generated in order to summarise the overall differences among

276 treatments. Gene Ontology mapping and functional domain analysis were performed using
277 Blast2GO (v.4.1.9). The mass spectrometry proteomics data have been deposited to the
278 ProteomeXchange Consortium via the PRIDE (Vizcaíno et al. 2016) partner repository with
279 the dataset identifier PXD011567.
280
281 *2.7. Statistical data analyses*
282 Analysis of variance (ANOVA) was done using the R environment (R v3.1.3; R core team
283 2015) to test the null hypotheses that there would be no difference in the (i) tenacity (ii)
284 number of byssal threads (with n = 5) and (iii) relative (post imputation) abundances of
285 individual proteins (with n = 4). The data were screened for normality (q-q plots, and Shapiro-
286 Wilk tests) and homogeneity of variance (Levene's test, using the *car* package; Fox and
287 Weisberg 2011) to ascertain assumptions for ANOVA. Pairwise comparisons were computed
288 when the main test was significant. Statistical significance was assumed at $\alpha = 0.05$. In order
289 to explore the possible relationships between the effects of microplastics on protein
290 expression and effects on the biological functioning of blue mussels, Pearson's R correlation
291 coefficients were calculated.
292 PERMANOVA was performed on post imputation data to test the null hypothesis that the
293 haemolymph proteomes (composition and relative abundances of all 216 identified proteins)
294 of mussels exposed to PLA or HDPE microplastics or to no microplastics (controls) would
295 not differ. Patterns of difference in the haemolymph proteomes were visualized with
296 canonical analysis of principal coordinates based on Bray-Curtis resemblance matrices (using
297 PRIMER V6.1.12 with PERMANOVA+).
298
299 **3. Results**
300 *3.1. Effects of microplastics exposure on biological functions of* M. edulis

After 52 days of exposure to microplastics, the tenacity was lower ($F_{2,12}$= 5.6, P = 0.019) and there were fewer byssal threads ($F_{2,12}$= 10.3, P = 0.002) produced by mussels exposed to HDPE microplastics compared with mussels exposed to PLA or to no microplastics (Figure 1). Specifically, after exposure to HDPE, attachment strength of *M. edulis* was ~3.0 times less compared to the controls, while it was ~2.2 times less compared with mussels exposed to PLA (Figure 1a). Mirroring this, the number of byssal threads produced by mussels exposed to HDPE was ~1.6 times less compared to the controls and ~1.8 times less compared to those exposed to PLA (Figure 1b). Across all treatments, there was a significant positive relationship between attachment strength and the number of byssal threads (Pearson's r = 0.78, t = 4.46, P < 0.001).

3.2. Effects of microplastics on the haemolymph proteome of M. edulis

High resolution quantitative mass spectrometry identified 2,745 peptides, representing 216 high confidence proteins from *M. edulis* (Table S1). The haemolymph proteome was significantly altered by microplastics (*pseudo*-$F_{2,9}$ = 2.89, P = 0.001), with those exposed to HDPE or to PLA having significantly different protein profiles compared to those not exposed to microplastics (HDPE vs Control: *pseudo*-t = 1.91, P = 0.023 and PLA vs Control: *pseudo*-t = 1.48, P = 0.029). The haemolymph proteome of mussels exposed to HDPE also differed to those exposed to PLA (HDPE vs PLA: *pseudo*-t = 1.67, P = 0.030). This is illustrated by ordination using canonical analysis of principal coordinates (Figure 2). Furthermore, ~19% of the haemolymph proteome (40 proteins) was significantly affected by exposure to microplastics, with the majority being expressed at greater abundances (Table 1) when microplastics were present, compared to the control.

Of the 40 statistically significant proteins, six were differentially expressed in mussels exposed to both types of microplastic compared with controls. A further 11 were differentially expressed only in those exposed to HDPE compared with controls and four were differentially expressed only in those exposed to PLA compared with controls. Nine proteins differed between HDPE and PLA while five proteins differed in expression between HDPE-exposed and both PLA-exposed and control mussels (Table 1, Figure 3). Post-hoc tests were unable to determine the differences in the remaining five proteins. Functional annotation through homology searches and gene ontology mapping assigned functional information for statistically significant differentially expressed proteins identifying proteins with putative roles in the immune system, metabolism and detoxification, as well proteins of unknown function (Table 1).

3.2.1. *Effects of microplastics exposure on the mussel immune complement*

Within the haemolymph of mussels exposed to microplastics, 11 putative immune-responsive proteins were differentially expressed. Exposure to HDPE resulted in increased abundance of four immune-responsive proteins, including a putative proinflammatory cytokine, macrophage migration inhibitory factor (HE609105.1), as well as three complement C1q domain-containing (C1qDC) proteins (FR715598.1; FR715581.1; HE609753.1) (Table 1, Figure 4). One of these putative C1qDC proteins (FR715598.1) was also increased in mussels exposed to PLA in comparison to the control. In addition, both microplastics treatments increased the abundance of a fibrinogen-related protein (OPL33687.1). In contrast, a second fibrinogen-related protein (OPL32613.1) was reduced within both treatments in comparison to control. Six immune proteins were differentially expressed between mussels exposed to PLA compared with those exposed to HDPE. In comparison to PLA, exposure to HDPE resulted in increased abundance of a putative pore-forming apextrin-like protein (HQ709238.1), a galectin

(AJQ21509.1), as well as a putative antimicrobial peptide, mytimycin precursor (AET85056.1). In contrast, PLA exposure increased the abundance of two C1qDC proteins (FR715612.1; HE609604.1) in comparison to HDPE.

3.2.2. Effects of microplastics exposure on the abundance of metabolic proteins

Seven putative metabolic proteins were differentially expressed in response to one or both microplastics treatments. HDPE exposure resulted in increased expression of a glyceraldehyde-3-phosphate dehydrogenase (GAEN01008281.1), an aminopeptidase (GAEN01005918.1) and a protein putatively involved in retinal metabolism, retinol dehydrogenase 1 (OPL33362.1) (Table 1, Figure 4). Two putative metabolic enzymes, a putative aspartate cytoplasmic protein (HE662841.1) and phosphoglycerate kinase (GAEM01000061.1) were increased within mussels exposed to PLA in comparison to both control and HDPE individuals. PLA exposure also resulted in a reduction in a metalloproteinase inhibitor (GAEM01005782.1) in comparison to mussels exposed to HDPE. Both HDPE and PLA also led to an elevated abundance of a putative peptidyl-prolyl cis-trans isomerase protein (GAEN01009083.1) compared with control mussels.

3.2.3. Potential detoxification proteins altered in response to microplastics exposure

Four proteins previously identified to have putative roles as biomarkers of detoxification within molluscs were differentially expressed in response to microplastics exposure. Exposure to either microplastics treatment resulted in the increased expression of a putative heavy metal binding protein (HE609570.1) and putative detoxification enzyme, deferrochelatase peroxidase (GAEN01007747.1) in comparison to control mussels. Furthermore, a ferritin heavy oocyte (GAEN01007405.1) was increased in response to HDPE exposure in comparison to control mussels. A fourth protein, with a putative role in

detoxification (cathepsin D; GAEM01006053.1), was also identified to be significantly affected by microplastics but post-hoc tests were not significant.

3.2.4. Additional biomarkers associated with microplastics exposure

Aside from variation in immune, metabolic and detoxification proteins, microplastics exposure changed the abundance of an additional 19 proteins. Both HDPE and PLA reduced the abundance of a protein of unknown function (OPL21291.1) compared with control mussels. Exposure to HDPE increased the expression of proteins involved in a variety of biological processes, including neurogenesis (GAEM01003123.1; OPL21044.1), structural integrity (GAEM01002086.1; GAEM01005782.1; OPL21594.1), DNA binding (CAD37821.1; CAC94907.1; GAEN01008605.1), and proteins of unknown function (OPL32817.1) in comparison to control and/or PLA treatment. In contrast, HDPE reduced the abundance of two proteins with roles in structural integrity (HE662833.1, GAEN01011200.1).

Exposure to PLA increased the expression of a putative growth factor protein (GAEN01008261.1) and a protein of unknown function (HE609843.1) in comparison to control and HDPE-exposed individuals, respectively. One putative titin-like protein (GAEN01023435.1) was reduced within mussels exposed to PLA in comparison to control mussels. Post-hoc tests were unable to determine the direction of differences in four additional proteins found to be significantly affected, including proteins involved in structural integrity (GAEN01011004.1; GAEN01007066.1), translation (GAEN01008711.1) and a protein of unknown function (GAEN01005668.1).

4. Discussion

After long-term (52 days) exposure to ~1 particle mL^{-1} of HDPE microplastics, the number of byssal threads produced and the tenacity of *M. edulis* were reduced by approximately 50% when compared with mussels not exposed to microplastics. Tenacity is paramount to the ability of mussels to form and maintain reefs without being dislodged by hydrodynamic forces (Bell and Gosline, 1997). The ability to produce byssal threads and to form aggregations also increases fertilization success, makes mussels more resistant to predation and, overall, increases the probability of their survival (Christensen et al, 2015). Weakened attachment strength in response to conventional microplastics could, therefore, result in cascading ecological (by reducing the habitat availability for intertidal communities that depend on mussel reefs) and economic (by reducing yields of suspension culturing of mussels in aquaculture) consequences. For example, the mussel aquaculture industry is already worth ~3-4 billion USD globally per year (FAO 2015) and is expected to grow in the coming decades. Reductions in the tenacity of two similar species of mussel, *Mytilus trossulus* (O'Donnell et al. 2013) and *Mytilus coruscus* (Zhao et al. 2017) have also been found in response to ocean acidification. Given that the concentrations of microplastics in the oceans (Jambeck et al. 2015) and the acidity of seawater (IPCC 2014) are both likely to increase in the coming decades, future research should assess their combined effect on the health and tenacity of mussels.

In order to complement the measures of tenacity and to provide a detailed assessment of mussel health in response to microplastics exposure, we assessed changes in the proteome of the mussel haemolymph. We chose the haemolymph because it plays an important role as a crucial transporter of nutrients and oxygen, as well as being a primary site of immune activity and xenobiotic detoxification (Malagoli et al. 2007). Information regarding the effects of microplastic exposure on an organism's proteome are limited, but Sussarellu et al. (2016) found

424 that exposure to polystyrene microplastics altered proteins in the oocytes of oysters and that
425 this corresponded to a reduction in fertility. In the current study, exposure to HDPE or PLA
426 microplastics resulted in complex changes in a number of key biological processes, including
427 immunity, metabolism and detoxification.
428
429 Exposure of mussels to either PLA or HDPE microplastics resulted in changes in the
430 immunological profiles of their haemolymph. The immune system represents an important
431 obstacle to infection and disease, and has been extensively studied and characterised in mussels
432 (Campos et al. 2015; Wu et al. 2016) and while interactions between microplastics and aspects
433 of the mussel cellular immune response have been previously documented (von Moos et al.
434 2012; Avio et al. 2015; Paul-Pont et al. 2016), here we provide evidence of changes within the
435 humoral components of the mussel haemolymph in response to microplastics exposure.
436 Specifically, members of the C1qDC protein family were affected by exposure to
437 microplastics. C1qDC genes function in pathogen recognition (Gerdol et al. 2011) with certain
438 genes elevated in response to bacterial challenge (Gestal et al. 2010). Additionally, two
439 pathogen recognition molecules, galectin-2 and apextrin were upregulated in mussels exposed
440 to HDPE compared with PLA microplastics. Within molluscs, galectin-2 and apextrin have
441 been characterised to promote phagocytosis (Vasta et al. 2015) and function in membrane pore
442 formation (Estevez-Calvar et al. 2011) respectively. Other effector molecules altered by
443 microplastics exposure, included an increase in the antimicrobial peptide, myticin (Mitta et al.
444 1999), while fibrinogen-related proteins, functional in antigen recognition (Romero et al.
445 2011), were either up- or down- regulated in response to microplastics exposure. These
446 immunological changes may be due to physical abrasion from the microplastics after being
447 ingested by the mussels. Previous research by Avio et al. (2015) on a similar species of mussel
448 to those used in the current study, *Mytilus galloprovincialis*, found similar immunological

449 responses to virgin and contaminated microplastics (polyethylene and polystyrene) thus
450 suggesting physical abrasion as the cause of response. It is possible that microplastics can
451 translocate into tissues such as the gills and digestive tract (von Moos et al. 2012; Avio et al.
452 2015; Paul-Pont et al. 2016), as well as the haemolymph (Browne et al. 2008; Avio et al. 2015).
453 Although microplastics in the haemolymph were not quantified in the present study, it is
454 possible that physical abrasion of the tissue may have triggered the observed immunological
455 responses but further research is required.
456
457 Despite some differences in the effects of PLA and HDPE on the proteome, a number of
458 proteins responded similarly to both types of microplastic including complement C1q domain-
459 containing proteins (discussed above) and detoxification proteins, such as a peroxidase and a
460 heavy metal-binding protein. Aside from direct immunological activity, immune-responsive
461 proteins within the haemolymph may have roles in detoxification. Within *Mytilus* species,
462 Cq1DC protein expression has been identified to change in response to heavy metal exposure
463 suggesting a role in detoxification (Liu et al. 2014). Within the present study, a heavy metal
464 binding protein, which contained a complement-like domain, was increased in response to both
465 types of microplastics, indicating a potential conserved biomarker of microplastics exposure.
466 Heavy metal-binding proteins with strong reactivity to metal pollutants have been previously
467 characterised within the mussel haemolymph (Renwrantz and Werner 2007). While organisms
468 require metal ions in trace amounts, excessive quantities can be toxic (Mejáre and Bülow 2001)
469 and require removal. Changes in immune proteins, as well as metal binding proteins, have been
470 characterized previously in mussels in response to other pollutants, highlighting the role of the
471 immune response as an indicator of a stressed phenotype (Coles et al. 1995). The ability of
472 mussels to detoxify microplastics and associated by-products has been investigated through the
473 measurement of detoxification enzyme activity (Paul-Pont et al. 2016), transcriptional

474 responses in antioxidant genes (Avio et al. 2015), as well as the assessment of oxidative damage
475 within exposed individuals (von Moos et al. 2012; Paul-Pont et al. 2016). We identified one
476 such detoxification enzyme, a peroxidase, to be increased within both microplastic treatments.
477 Peroxidases are vital enzymes involved in the degradation of by-products of respiration
478 (Brigelius-Flohé and Maiorino 2013) and have been identified to have increased enzyme
479 activity within mussels in response to exposure to other pollutants (Vidal-Liñán et al. 2015)
480 and within the marine copepod, *Paracyclopina nana*, in response to microplastics (Jeong et al.
481 2017). While the exact role of this peroxidase within *Mytilus* is unknown (Tomanek 2015), it
482 has previously been found to change in abundance in response to fluctuations in temperature,
483 which suggests a role in oxidative stress (Fields et al. 2012). The generation of immune and
484 detoxification defenses can be metabolically costly, placing additional demands on a stressed
485 host. Within mussels, exposure to microplastics can affect metabolic enzymes, involved in
486 essential processes, such as energy metabolism and respiration. For example, metabolic
487 enzymes involved in glycolysis have been found to increase in response to microplastics
488 exposure, which has been suggested to be associated with mounting a detoxification response
489 (Paul-Pont et al. 2016). In the current experiment, the filtration rates of *M. edulis* were reduced
490 by exposure to either HDPE or PLA microplastics, compared with controls (results reported in
491 Green et al. 2017). Other contaminants can also cause similar responses, for example, in
492 response to anthracene, Mediterranean clams (*Ruditapes decussatus*) also had reduced
493 filtration rates and altered proteomes (Sellami et al. 2015). The reduction in the abundance of
494 metabolic proteins may, therefore, be associated with reduced feeding but further research is
495 needed to establish this causal link.
496
497 Myosin was reduced within mussels exposed to HDPE compared with controls. Myosin is
498 involved in generating muscle contraction in bivalves (Yamada et al. 2000) and has been found

499 to be altered by exposure to silver nanoparticles in a related mussel, *M. galloprovincialis*
500 (Gomes et al. 2013). The deficiency in myosin, coupled with the expression of immune and
501 detoxification proteins associated with mussels exposed to HDPE microplastics, may have
502 contributed to less byssal threads being secreted. The tenacity of mussels is primarily related
503 to byssal threads, either based on the number of threads or to their thickness (Carrington 2002).
504 The byssus proteome of another marine mussel of the same genus, *M. coruscus*, has been
505 previously characterised and a selection of other structural proteins (collagen-like) were
506 identified (Qin et al. 2016) and suggested to provide adjustable tension allowing for stable
507 attachment within dynamic rocky intertidal environments (Qin and Waite 1995).
508
509 Within the present study, exposure to conventional microplastics, HDPE, as well as a
510 biodegradable alternative, PLA, resulted in changes to the haemolymph proteome, including
511 proteins associated with stressed phenotypes. Certain proteins involved in immunity and
512 detoxification, affected by both microplastics, provide candidate biomarkers for further
513 research. Overall HDPE alone resulted in more proteomic changes in comparison to PLA.
514 Despite being less severe, the effects of PLA microplastics on the proteome of *M. edulis*
515 provides additional support to the growing body of literature on the potential issues of
516 biodegradable alternatives. For example, PLA microplastics have also been found to reduce
517 the biodiversity and abundance of organisms in marine invertebrate communities (Green 2016)
518 and to decrease the biomass of benthic primary producers (Green et al. 2017; Green et al. 2016).
519 Biodegradable plastics are set to become more dominant as packaging in the future, possibly
520 replacing some conventional plastics (Markets and Markets 2015). They are, therefore, also
521 more likely to become litter. Current testing methods, even those developed specifically for
522 marine habitats (ASTM D7991-15), are limited in their ability to predict the break-down and
523 ecological impacts of biodegradable plastics in the real world (Bioplastics Europe 2016).

524 Multidisciplinary research combining molecular, ecophysiological and traditional ecological
525 techniques is recommended in order to gain a more holistic understanding of the potential
526 impacts of conventional and biodegradable polymers.
527
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Table 1. Proteins in *M. edulis* haemolymph with significantly different relative abundances in individuals exposed to HDPE, PLA microplastics or to no microplastics (CONT). Fold differences are indicated by "δfold", and are in **bold** when significantly different according to post-hoc tests. ANOVA (with F and P values) and pairwise post-hoc tests with *****indicating significant differences at P<0.05). The source of the information on protein annotation is detailed in Table S2.

	Protein ID	Protein Annotation	F	P	δfold HDPE vs CONT	δfold PLA vs CONT	δfold HDPE vs PLA
Immune	FR715598.1	C1Q Domain Containing 1Q19	80.2	<0.001	**7.0***	**5.4***	1.5
	HE609753.1	Complement C1Q 4	58.0	<0.001	**4.2***	-0.1	**4.4***
	OPL33687.1	Fibrinogen-Related	8.0	0.009	**3.7***	**3.6***	0.1
	AJQ21509.1	Galectin 2	6.7	0.016	0.7	-0.5	**1.2***
	HQ709238.1	Apextrin	5.6	0.026	1.9	-0.9	**2.8***
	FR715612.1	C1Q Domain Containing 1Q33	5.5	0.027	2.3	-1.5	**3.8***
	HE609604.1	Complement C1Q 2	5.1	0.032	-2.8	1.3	**-4.1***
	FR715581.1	Complement C1Q Tumor Necrosis Factor	4.7	0.039	**3.8***	2.6	1.1
	HE609105.1	Macrophage Migration Inhibitory Factor	4.5	0.045	**0.8***	0.3	0.5
	AET85056.1	Mytimycin precursor	4.3	0.049	1.9	-3.3	**5.1***
	OPL32613.1	Microfibril-Associated Glyco 4	39.3	<0.001	**-4.0***	**-5.2***	1.3
Metabolism	GAEN01005918.1	Aminopeptidase N	24.6	<0.001	**3.3***	0.6	**2.7***
	HE662841.1	Aspartate Cytoplasmic	9.0	0.007	0.0	**0.7***	**-0.7***
	GAEN01009083.1	Peptidyl-Prolyl Cis-Trans Isomerase	6.8	0.016	**0.6***	**0.5***	0.1
	GAEN01008281.1	Glyceraldehyde-3-Phosphate Dehydrogenase	6.3	0.020	**0.5***	0.4	0.2
	GAEM01005782.1	Metallo Ase Inhibitor 3	5.4	0.029	1.5	-0.5	**2.0***
	GAEM01000061.1	Phosphoglycerate Kinase	4.9	0.037	0.3	**0.6***	-0.3
	GAEM01005782.1	Retinal Dehydrogenase 1	5.8	0.024	**1.9***	1.4	0.5
Structural	GAEM01002086.1	Shell -5	12.5	0.002	**2.8***	1.2	1.6
	HE662833.1	Calponin -1	6.4	0.019	**-3.1***	-1.8	-1.3
	GAEN01011200.1	Myosin Essential Light Chain	6.1	0.021	**-1.2***	-0.8	-0.4
	OPL21594.1	Singed Isoform X3	4.8	0.038	**0.6***	0.5	0.2
	GAEN01011004.1	Myosin Heavy Non-Muscle-Like	4.6	0.043	2.1	2.0	0.1
	GAEN01023435.1	Titin-Like	4.5	0.045	-0.5	**-0.7***	0.2
	GAEN01007066.1	Myosin Regulatory Light Chain Smooth Adductor Muscle-Like Isoform X3	4.4	0.047	-1.3	-1.1	-0.2
Detoxification	HE609570.1	Heavy Metal-Binding Protein	10.5	0.004	**4.4***	**2.9***	1.5
	GAEN01007747.1	Probable Deferrochelatase Peroxidase	7.2	0.013	**2.3***	**2.5***	-0.2
	GAEM01006053.1	Cathepsin D	4.6	0.042	2.2	2.2	0.0

	GAEN01007405.1	Ferritin Heavy Oocyte Isoform	4.8	0.038	**2.3***	1.7	0.6
DNA binding	CAD37821.1	Histone H2A	8.3	0.009	**2.9***	-1.0	**3.9***
	GAEN01008605.1	Cytoplasmic A3A	6.8	0.016	**0.8***	0.7	0.2
	CAC94907.1	Histone H1	4.4	0.046	**0.8***	0.2	0.5
Translation	GAEN01008711.1	60S Ribosomal L7	4.3	0.047	-1.5	-0.2	-1.4
Unknown	OPL21291.1	Hypothetical Protein Am593_06205	8.8	0.007	**-0.9***	**-0.8***	-0.2
	HE609843.1	Alpha-Crystallin A Chai	5.2	0.031	-0.9	0.3	**-1.1***
	OPL32817.1	Hypothetical Protein Am593_04753	4.6	0.041	1.1	-2.1	**3.1***
	GAEN01005668.1	Hypothetical Protein Brafldraft_84721	4.3	0.048	1.1	1.0	0.2
	GAEM01003123.1	Fatty Acid-Binding Brain	58.6	<0.001	**4.7***	-0.1	**4.8***
	GAEN01008261.1	Hypothetical Conserved 327	7.3	0.013	1.0	**3.0***	-2.0
	OPL21044.1	Atrial Natriuretic Peptide Receptor A	12.7	0.002	**4.9***	1.2	**3.7***

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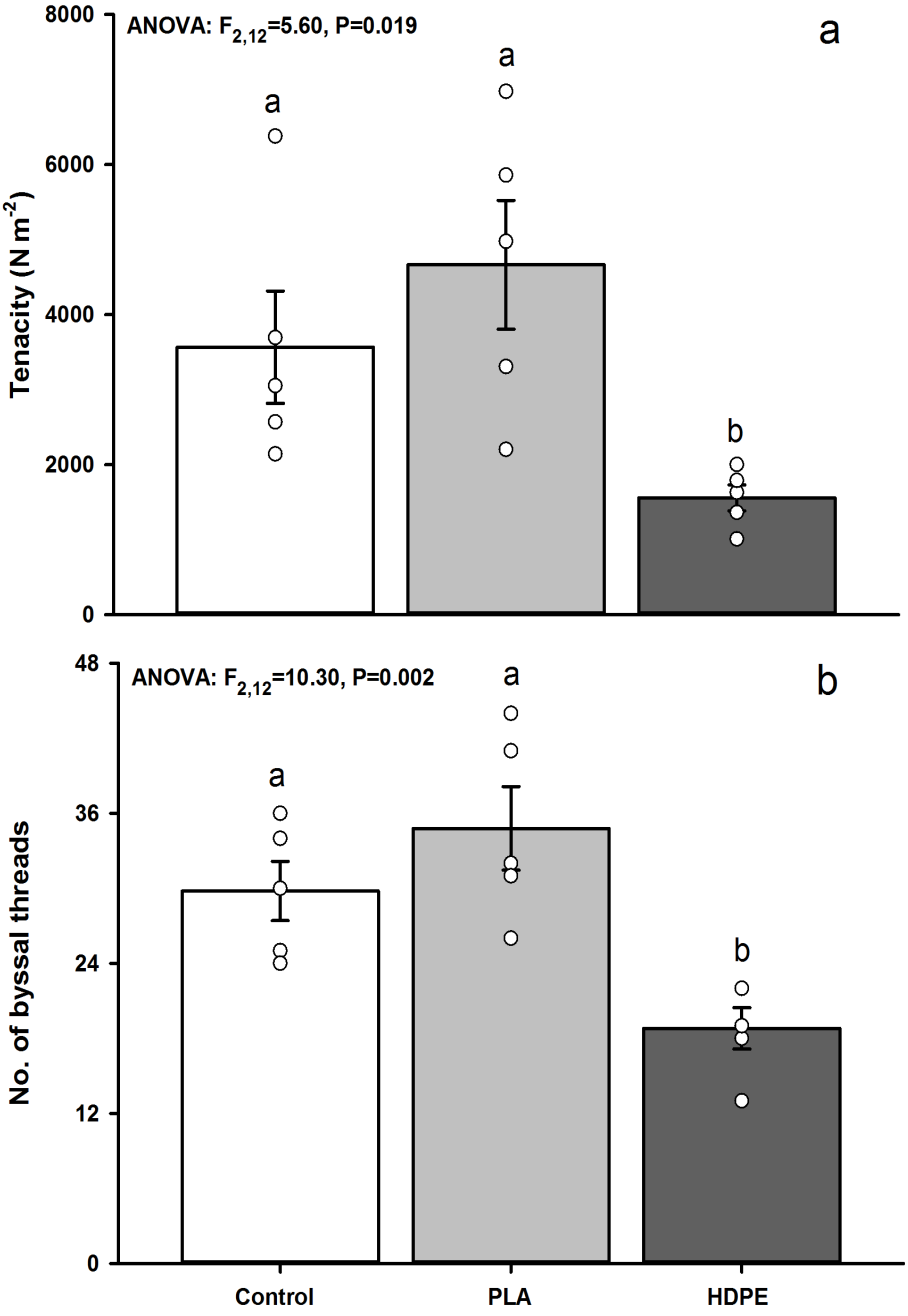
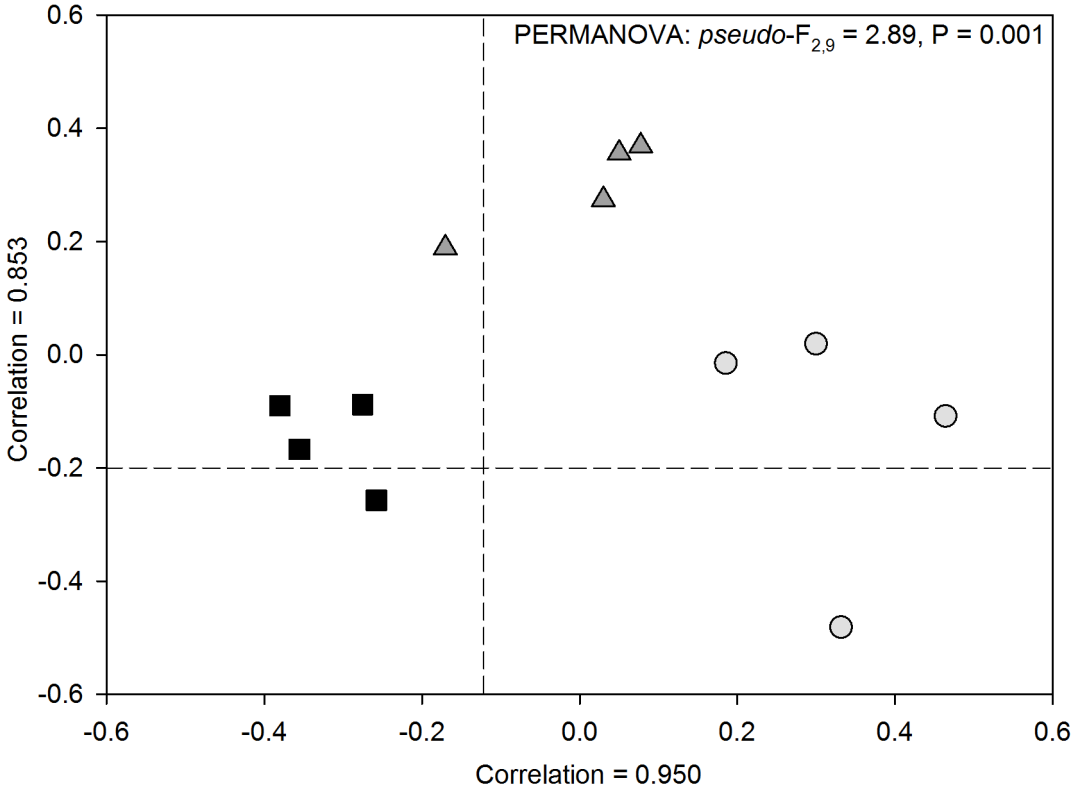


Figure 1. Mean (±S.E.M.) tenacity (a) and number of byssal threads (b) of *M. edulis* after 52 days of repeated exposure to 25 µg L^{-1} of PLA (polylactic acid) or HDPE (high-density polyethylene), or no microplastics (control). Letters denote significantly different treatments determined by posthoc tests (Tukey's).



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824 **Figure 2.** Canonical analysis of principal coordinates (m=5, 75% of samples correctly
825 allocated) of the composition and structure of haemolymph proteomes (using all 216
826 identified proteins) from *M. edulis* after 52 days of repeated exposure to 25 µg L^{-1} HDPE
827 (high density polyethylene) microplastics (■), PLA (polylactic acid) microplastics (▲), or
828 no microplastics (○).

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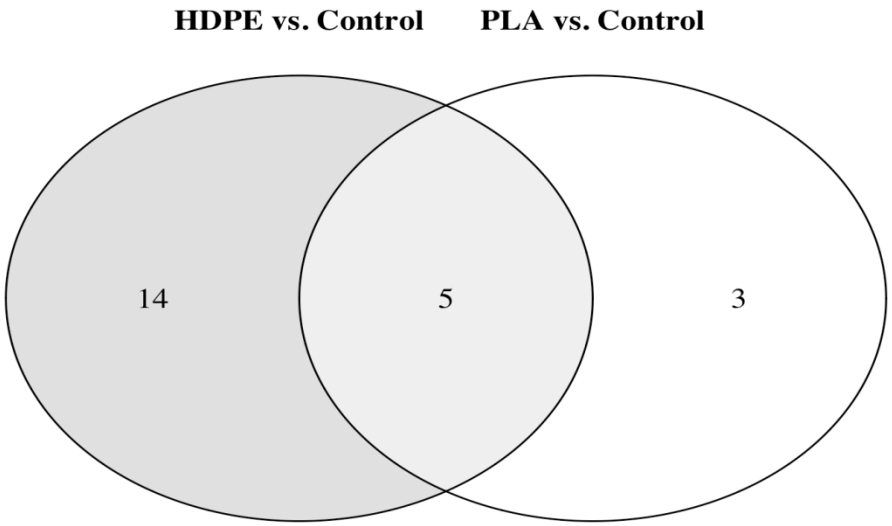
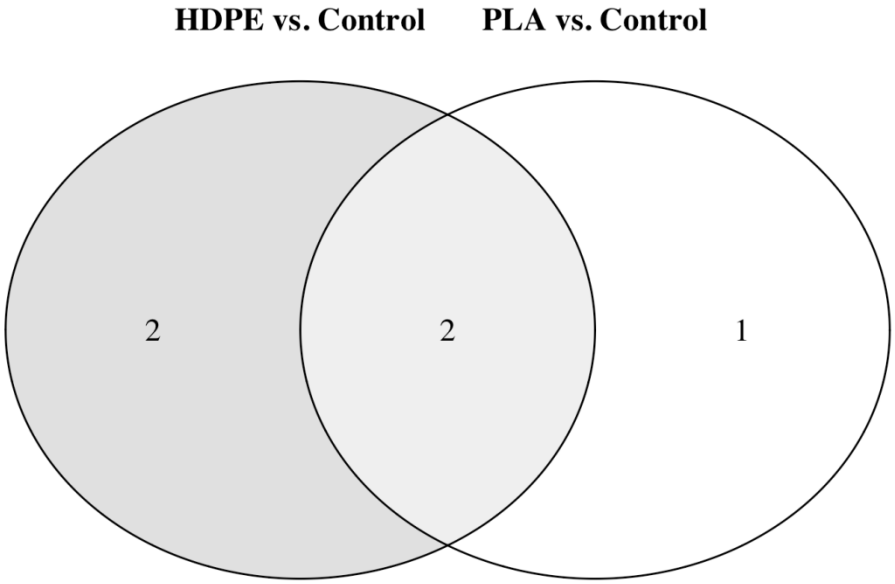
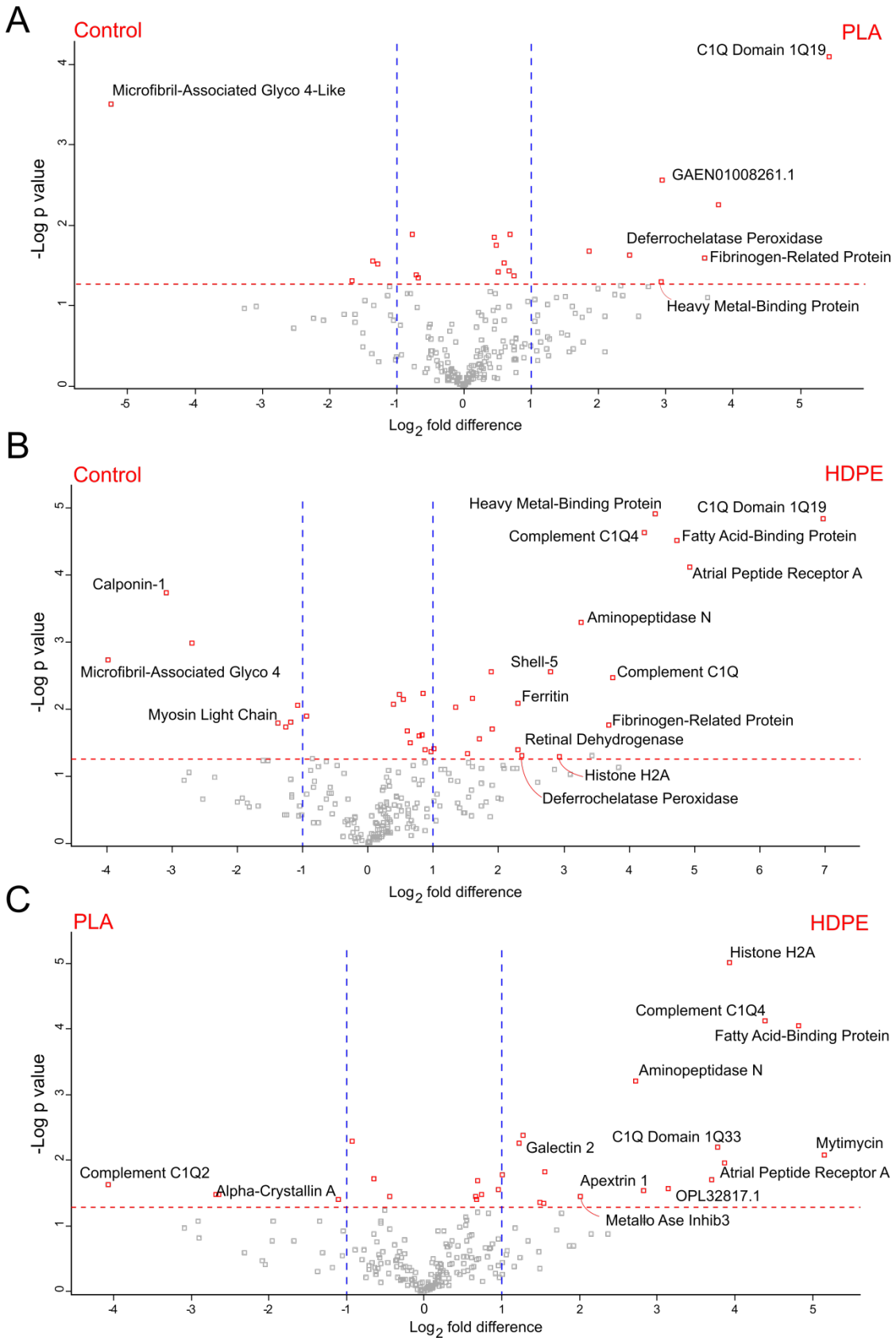


837 **(A)**



838 **(B)**

839 **Figure 3.** Venn Diagram depicting the number of proteins in mussel haemolymph altered in
840 abundance by exposure to either HDPE or PLA microplastics. For both treatments, the
841 number of proteins with A) increased expression, or B) decreased expression relative to
842 control treatment is shown.



843

844 **Figure 4.** Volcano plots based on post-imputation relative abundance of all identified proteins

845 comparing A) Control versus PLA, B) Control versus HDPE and C) PLA versus HDPE. Each

symbol represents a specific protein with those above the red line being considered statistically significant (p-value <0.05 based on post-hoc tests after ANOVA) and those to the right and left of the vertical lines indicate relative fold changes of ≥ 2.

Supporting Information

Table S1: 216 proteins identified with high confidence in the haemolymph of control and HDPE- and PLA-exposed *M. edulis*. Normalised label free quantitative (LFQ) intensities were determined for four biological replicates for each group and log2 transformed. Missing LFQ values were replaced through imputation. PEP, peptide error probability; MS/MS, number of second mass spectra determined for each peptide. Additional protein annotations were obtained by BLAST2Go analysis.